MICROBIOLOGICAL REVIEWS, Dec. 1994, p. 616–630
0146-0749/94/$04.00+0

Vol. 58, No. 4

Chemostat Cultivation as a Tool for Studies on Sugar Transport in Yeasts

RUUD A. WEUSTHUIS,[1] JACK T. PRONK,[1] PETER J. A. VAN DEN BROEK,[2] AND JOHANNES P. VAN DIJKEN[1]*

Department of Microbiology and Enzymology, Kluyver Laboratory of Biotechnology, Delft University of Technology, 2628 BC Delft,[1] and Department of Medical Biochemistry, Sylvius Laboratory, State University of Leiden, 2333 AL Leiden,[2] The Netherlands

INTRODUCTION

All yeasts presently known are able to utilize one or more sugars as their principal source of carbon and energy (3–5). Many yeast strains that are commonly used in biotechnological processes have been obtained from natural habitats with high sugar concentrations, in which they rapidly convert the available sugars to ethanol. In these ecosystems, the growth rate of yeasts like *Saccharomyces cerevisiae* (baker's yeast, brewer's yeast) may be limited by the availability of nutrients other than the sugar carbon source. Under such conditions, their competitiveness is not determined by affinity for the sugar substrate or the energetic efficiency of sugar utilization. This is reflected by the characteristics of their sugar uptake systems, which generally have a rather poor substrate saturation constant for the sugar substrate: K_m values are usually in the 10^{-3} to 10^{-2} M range (111). Yet, at saturating sugar concentrations, glycolytic fluxes in these yeasts can attain very high values. This is a bonus in the classical biotechnological applications of *S. cerevisiae*, i.e., during the leavening of dough and the production of alcoholic beverages, for which a high specific rate of alcohol production is desired.

The Crabtree-positive yeasts, which include *S. cerevisiae*, have a strong tendency toward alcoholic fermentation. In these yeasts, high rates of sugar uptake result in alcoholic fermentation, even when oxygen is present in excess (50, 79, 106). In modern large-scale production processes, e.g., for the production of baker's yeast, single-cell protein, or heterologous proteins, alcoholic fermentation is not desired, because it inhibits growth and reduces the biomass yield (49). In these processes, alcoholic fermentation is avoided by feeding sugar to the cultures at a low rate in a fed-batch mode. This results in a low concentration of sugar in the culture and, consequently, in a low rate of sugar uptake. At these low uptake rates, sugar metabolism is fully respiratory (79, 106). Consequently, high biomass yields are obtained and accumulation of toxic products is prevented.

The high K_m values for sugar transport that are characteristically found in *S. cerevisiae* strains are not typical for all yeasts: many species appear to be well equipped for growth at

* Corresponding author. Mailing address: Department of Microbiology and Enzymology, Kluyver Laboratory of Biotechnology, Delft University of Technology, Julianalaan 67, 2628 BC Delft, The Netherlands. Phone: (31) 15 782387. Fax: (31) 15 782355.

TABLE 1. Sugar transport parameters for various yeasts[a]

Yeast	Fold accumulation of 6-deoxyglucose[b]	K_m (mM)[c]	V_{max} (mmol/g/h)[a]	C_s (μM)[d]
Crabtree-positive yeasts				
Saccharomyces cerevisiae	0.5	1.0	12	110
		20	9	
Schizosaccharomyces pombe	1.1	1.5	9.0	160
Torulopsis glabrata	0.5	1.2	31	100
		18	93	
Crabtree-negative yeasts				
Candida utilis	70	0.025	8.4	5
		0.2	5.4	
Pichia stipitis	190	0.015	6.0	5
Kluyveromyces marxianus	110	0.025	1.2	35
		1.8	2.0	

[a] Data from reference 111.
[b] Accumulation of 6-[^{3}H]deoxyglucose was measured.
[c] Determined in 10-s incubations with D-[U-^{14}C]glucose. In many cases, biphasic Hanes plots were obtained. This is indicated by the presence of two apparent K_m and V_{max} values. However, the reported values are crude estimates, since they were obtained by simple linear rather than nonlinear regression. Values are for suspensions of yeasts pregrown in aerobic, glucose-limited chemostat cultures at $D = 0.10\ h^{-1}$.
[d] Determined by rapid sampling of cultures into liquid nitrogen. For conditions, see footnote *c*.

low sugar concentrations and are able to synthesize transport systems with K_m values of 10^{-5} minus 10^{-4} M (111) (Table 1).

Transport constitutes the first step in the metabolism of a large number of sugars (a notable exception being the metabolism of some oligosaccharides that are hydrolyzed outside the cell). As such, sugar transport is likely to have a substantial impact on the regulation of the glycolytic flux as a whole. In this respect, it has been suggested that sugar uptake is a rate-limiting step in glycolysis (24, 40, 56, 110). Because of its impact on the ecology and biotechnological applications of yeasts, sugar transport by yeasts has been the subject of a large number of studies. The majority of these have been performed with samples from shake-flask cultures grown in the presence of excess sugar. Unfortunately, such cultures are poor model systems, since they exhibit a number of inherent drawbacks for quantitative studies on sugar transport in yeasts. The aim of this paper is to review the applicability of chemostat cultivation as a tool for studies on quantitative aspects of sugar transport in yeasts. Particular attention will be paid to the relation between the kinetics of sugar transport in cell suspensions, as determined in experiments with radiolabeled sugars, and the kinetics of sugar utilization observed in growing cultures.

MECHANISMS OF SUGAR TRANSPORT

Since sugars are highly polar molecules, free diffusion across the membrane lipid bilayer probably does not contribute significantly to their rate of entrance into the cell at low sugar concentrations (57). However, at high sugar concentrations, as for instance in grape juice, free diffusion might contribute to some extent to the overall sugar influx (39, 117).

Facilitated-diffusion systems, in which translocation of the sugar across the membrane is mediated by a carrier protein, are widespread among yeasts (3, 4, 55). In the case of facilitated diffusion, the driving force for solute translocation is provided exclusively by the concentration gradient of the solute over the membrane. Therefore, uptake of sugars by facilitated diffusion does not require metabolic energy. Since the driving force for sugar uptake becomes zero when internal and external solute concentrations are equal, this process does

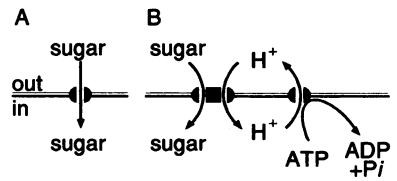

FIG. 1. Schematic representation of two mechanisms of sugar transport in yeasts. (A) Facilitated diffusion driven by the concentration gradient of the sugar. (B) Proton-sugar symport driven by the proton motive force and the sugar concentration gradient. ATP hydrolysis is required to expel the protons that enter the cytosolic compartment together with the sugar. For maltose transport in *S. cerevisiae*, it has been established that 1 mol of ATP is required per mol of sugar transported (121).

not allow the uptake of sugars against a concentration gradient. In particular, during growth at very low extracellular sugar concentrations, intracellular accumulation of sugars may be necessary to allow the cytoplasmic sugar kinases and disaccharide hydrolases to function optimally (55). This can be accomplished by coupling the uptake of a sugar molecule to the uptake of one or more protons via proton symport systems. Thus, the proton motive force over the plasma membrane can be used to drive intracellular accumulation of sugar. This proton motive force is generated mainly by the plasma-membrane H^+-ATPase complex, which couples the hydrolysis of ATP to ADP plus P_i to the outward translocation of protons (Fig. 1).

In many bacteria, sugar uptake and phosphorylation are tightly coupled. In the so-called group translocation systems (57), free intracellular sugar does not occur. Early studies on (deoxy)-glucose transport in *S. cerevisiae* have indicated that when sugar is added, sugar-phosphate appears faster in the cell than free glucose does, suggesting a similar mechanism for glucose transport in yeasts (37, 63, 109). The occurrence of a group translocation mechanism for sugar uptake in yeasts has, however, been a matter of dispute for many years (55). Recent studies on glucose uptake by *S. cerevisiae* have shown that free sugar can directly enter the cell, indicating that the glucose transporter catalyzes facilitated diffusion of free sugar (69). It is likely that the massive phosphorylation of the sugar, which is also observed with incubation times in the subsecond range (118), is caused by the excess sugar kinase activity in the cell or by a functional association of the transporter with intracellular sugar kinases as suggested previously (10, 11).

METHODS USED IN SUGAR TRANSPORT STUDIES

Transport Studies with Radiolabeled Sugars

The most widely applied method for studying uptake of sugars by suspensions of yeast cells is the use of radioactive (^{14}C- or ^{3}H-labeled) sugars. An inherent problem in transport studies with intact cells is the interference by subsequent metabolism. Nearly always, a significant decrease of the apparent uptake rate is observed within 15 s after addition of radiolabeled sugar (83). This effect is most probably caused by production of $^{14}CO_2$ and release of other labeled metabolites such as ethanol and organic acids. In practice, this problem can be reduced by using very short incubation times (5 to 10 s). Recent studies involving quenched-flow techniques (30) indicate that in starved galactose-grown *S. cerevisiae* cells, a 5-s uptake indeed gives a good indication of the initial influx rate (118). Under certain conditions however, e.g., in the presence of cyanide, transport was already leveling off after 0.2 s and

initial-influx measurements required sampling times in the subsecond time scale (118). This shows that the method to measure influx by the 5-s method should be used with care and has to be substituted in some cases by the rapid-quenching technique.

Interference of metabolism in uptake studies can be circumvented by the use of nonmetabolizable sugar analogs, such as the glucose analog 6-deoxyglucose (52, 90). Use of these compounds can provide only qualitative information, since kinetic parameters are likely to be different from those for the natural substrates. Nevertheless, when proper controls are included, the use of sugar analogs may yield important information on the uptake mechanism. For example, uptake of radioactive 6-deoxyglucose against a concentration gradient can be regarded as a reliable criterion for the presence of an energy-dependent uptake system (101, 111).

An obstacle inherent to transport studies with radioactive sugars is the nonspecific binding of labeled substrate to cellular components. Correction for nonspecific binding is a necessity since, especially in short-term uptake studies, it may contribute significantly to the cell-associated radioactivity. Various methods have been used to determine the contribution of binding to the total amount of radioactivity retained by the cells. These rely on inactivation of cells (e.g., by heat treatment [96]) or on control experiments performed at 0°C (10). Recently it was shown that significant reduction of binding, at least in *S. cerevisiae*, is best achieved by washing cells at −5°C with a solution containing 500 mM nonradioactive sugar (117).

Another pitfall in transport studies with radioactive sugars is the chemical impurity of many radiolabeled sugars. For example, commercially available preparations of D-[U-^{14}C]maltose may contain up to 2% glucose. Uptake of such impurities can contribute to a large extent to the total amount of radioactivity that is transported, in particular when the K_m for uptake of the impurity is much lower than that for the sugar of interest.

Proton Flux Measurements

In the case of H^+ symport mechanisms, sugar transport can also be determined indirectly by using a sensitive pH electrode to measure the alkalinization of weakly buffered cell suspensions upon addition of sugars (95). A major advantage of this method is that nonspecific binding does not interfere with the uptake assay. A disadvantage of the measurement of sugar-dependent pH changes is that these assays cannot be performed with standard growth media, which are usually strongly buffered. Furthermore, interference of metabolism with the transport assay can also be problematic since proton movements may be caused by various other metabolic processes as well, including ATPase activity and production of acidic metabolites. As mentioned above for radioactive uptake studies, interference of metabolism with alkalinization studies is reduced by measuring initial rates. If radioactive transport studies and alkalinization assays are performed under identical conditions, the fluxes obtained with these two methods can in principle be used to calculate sugar-proton stoichiometries.

In Vitro Sugar Transport Studies

Interference of sugar metabolism in transport studies is avoided by studying uptake in vitro with isolated membrane vesicles. Since cytoplasmic enzymes are absent in membrane vesicles, the transported sugar cannot be metabolized, thus facilitating calculations on uptake kinetics and accumulation ratios. Isolated yeast plasma membranes, however, do not form well-sealed vesicles (38), which is probably the reason that they do not exhibit carrier-mediated initial sugar influx. Nevertheless, this type of membrane vesicle can be used to measure counterflow (53). To decrease the leakiness of membrane vesicles, membranes have to be fused with artificial liposomes (38). In this way, membrane vesicles with transport parameters similar to those of intact cells can be obtained (71, 89). Studies on energy-dependent transport processes in yeast plasma membrane vesicles are complicated by the absence of a proton-translocating respiratory chain. The physiological membrane-energizing system, the plasma membrane ATPase complex, cannot be used to energize transport in right-side-out vesicles because its catalytic site is not accessible to ATP. These predicaments can be circumvented via introduction of a heterologous proton-translocating system. This can be accomplished by fusion of purified yeast plasma membrane vesicles with liposomes containing proton-pumping bovine heart cytochrome *c* oxidase (Fig. 2). In these fused vesicles, a proton motive force can be generated by addition of the artificial redox system ascorbate-TMPD (*N,N,N′,N′*-tetramethyl-*p*-phenylene diamine)-cytochrome *c* (105, 107, 108), which can be used to drive uphill transport of sugars as shown for galactose and lactose transport in *Kluyveromyces marxianus* (74, 107) and maltose transport in *S. cerevisiae* (105, 108). Although the fused-vesicle method allows detailed studies on the molecular mechanism and energy coupling of transport systems, it is not yet possible to quantitatively relate in vitro activities to in vivo uptake rates.

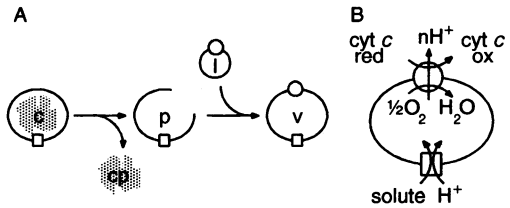

FIG. 2. In vitro sugar transport in hybrid plasma membrane vesicles. (A) Preparation of hybrids of cytochrome *c*-oxidase-containing liposomes and yeast plasma membrane vesicles. After rupturing yeast cells (c), cytoplasmic components (cp) are separated from plasma membranes (p) containing the sugar carrier (□). Membranes are fused with liposomes (l) containing cytochrome *c*-oxidase (○), resulting in the formation of closed vesicles (v). (B) Energization of active transport of a sugar in hybrid vesicles by the action of cytochrome *c* oxidase (cyt *c* ox), a primary proton pump capable of generating a proton motive force. For experimental details, see references 107 and 108.

Kinetic Analysis of Multicomponent Transport Systems

Studies on the kinetics of sugar uptake have in many cases revealed nonlinear Eadie-Hofstee or Hanes plots, indicating the activity of more than one transport system. When multiple transporters are active, the specific rate of sugar uptake (v) is described by equation 1.

$$v = \sum_{i=1}^{n} V_i = \sum_{i=1}^{n} \frac{V_{\max,i} C_s}{K_{m,i} + C_s} \quad (1)$$

In equation 1, $K_{m,i}$ is the apparent affinity constant of transport system i, $V_{\max,i}$ is the maximum capacity of transport system i, C_s is the substrate concentration, and n is the number of different transporters (103). In principle, more than two transporters can be operative simultaneously. In practice, curve fitting of nonlinear kinetics usually assumes the involvement of two kinetic components with different K_m values.

TABLE 2. Genes implicated in sugar transport in yeasts and length of the predicted amino acid sequences

Gene	Yeast	Substrate	Protein size (amino acids)	Reference
SNF3	*S. cerevisiae*	Glucose	884	16
HXT1	*S. cerevisiae*	Glucose	569	51, 58
HXT2	*S. cerevisiae*	Glucose	541	54
HXT3	*S. cerevisiae*	Glucose	567	51
HXT4	*S. cerevisiae*	Glucose	?	51
LGT1	*S. cerevisiae*	Glucose	576	88
GAL2	*S. cerevisiae*	Galactose	574	99
MAL61	*S. cerevisiae*	Maltose	614	19, 123
LAC12	*K. lactis*	Lactose	587	17
RAG1	*K. lactis*	Glucose	577	120

From equation 1 it follows that the various transporters contribute to the overall influx at all sugar concentrations. Therefore, as pointed out recently (39), kinetic constants cannot be obtained by a simple graphic analysis of linearized kinetic plots but should be obtained by dissection of the various kinetic systems by computer analysis (39, 85, 103, 117).

SUGAR TRANSPORT IN YEASTS

Monosaccharide Transport

The most intensively studied case of sugar transport in yeasts is that of glucose transport in *S. cerevisiae* (for a comprehensive review, see reference 55). Analysis of radiolabeled glucose uptake has indicated that, depending on the growth conditions, transport can be described by one or two kinetic components (43). Thus, high- and low-affinity uptake systems can be operative (10, 84). High-affinity glucose uptake, having an apparent affinity constant of about 1 mM, is most probably mediated by a facilitated-diffusion carrier, possibly in close association with a hexokinase (21). The low-affinity uptake component has been a matter of dispute. Originally it was estimated, from a direct analysis of biphasic Eadie-Hofstee plots, that this component would have a K_m of about 20 mM (10). Computer analysis of these data has, however, shown that the low-affinity part of the biphasic glucose uptake kinetics either has a very high K_m (in the molar range) or even may not exhibit saturation kinetics, which would indicate passive diffusion (39). Walsh et al. (117) have recently shown that most of this apparent low-affinity transport disappears when cells are quenched with high concentrations of nonradioactive sugar. This suggests that radioactive sugar can firmly bind to the cell surface and that improper washing of the cells leaves part of the sugar bound to the cells, thereby perturbing transport measurements. Nevertheless, even after proper correction for binding, these authors also reported biphasic transport curves, for which they suggested that deviations from linearity at high sugar concentration were caused by free diffusion. Moreover, they reported that carrier-mediated transport exhibited two different K_m values, namely, one in the 5 mM range and one in the 20 to 30 mM range.

Several *S. cerevisiae* mutants impaired in glucose transport activity have been isolated. Since none of these completely lacks the capacity to transport glucose, several genes are expected to code for glucose carriers. Indeed, a number of candidates have been identified, including *SNF3*, *HXT1* to *HXT4*, and *LGT1*, of which the last is probably identical to *HXT4* (Table 2). Each of these genes has sequence homology with mammalian glucose transporters. It is at present not clear whether all of these genes indeed code for a sugar transporter or whether they code for regulatory proteins, as has recently been suggested for *SNF3* (23). Identification of the structural genes encoding glucose carriers in *S. cerevisiae* will probably have to await purification of their gene products and reconstitution into in vitro systems.

A different type of glucose transport, mediated by a glucose-proton symporter, has been demonstrated in a number of non-*Saccharomyces* yeasts, including *Candida* spp. (83, 97), *Rhodotorula* spp. (46, 48), and *K. marxianus* (26, 41). In general, the apparent substrate saturation constants of these transporters are in the micromolar range. Such high-affinity glucose uptake systems are probably widespread among yeasts (25, 59) (Table 1).

The second-best-studied monosaccharide uptake system in yeasts is the galactose transporter. In *S. cerevisiae*, galactose can be transported by two systems, a constitutive low-affinity system with a K_m of more than 1 M and an inducible high-affinity transporter with a K_m of about 3 mM (89). It has been reported that galactose also induces a carrier with an apparent affinity of 340 mM (89). However, it is questionable whether this inducible low-affinity translocator exists or it is similar to the above-mentioned constitutive carrier with a K_m greater than 1 M, since the kinetic constants were obtained by direct analysis of Eadie-Hofstee plots and it can be expected that the real K_m value of the inducible low-affinity transporter will be higher (39). Inducible galactose transport is mediated by the *GAL2* gene product. *GAL2* encodes a protein with a predicted size of 574 amino acids (99), which most probably represents the high-affinity facilitated-diffusion galactose carrier. In contrast to *S. cerevisiae*, galactose transport in other yeasts can involve proton symport mechanisms (26). Data on galactose uptake in non-*Saccharomyces* yeasts are scarce, as is the case for other monosaccharide carriers, and will not be further discussed in this paper.

Disaccharide Transport

Glucose is by far the most commonly used substrate for fundamental physiological studies on sugar metabolism in yeasts. However, only few industrial applications are based on glucose as a feedstock. Industrial substrates such as molasses, whey, starch hydrolysates, and wort all contain disaccharides (sucrose, lactose, and maltose, respectively) as the major sugar component.

In contrast to glucose metabolism, disaccharide metabolism in yeasts is not necessarily initiated by uptake of the sugar molecule. For example, in the yeast *K. marxianus*, sucrose is initially hydrolyzed to glucose and fructose by the extracellular enzyme inulinase (91, 92). Subsequently, the component hexoses are transported into the cell (Fig. 3). Conversely, disaccharides can also be transported over the plasma membrane prior to hydrolysis by an intracellular hydrolase (Fig. 3). This is the case, for instance, for maltose utilization by *S. cerevisiae*. In *S. cerevisiae*, hydrolysis of sucrose can occur either intracellularly or extracellularly (65, 93). The general view, however, is that extracellular hydrolysis is the predominant route by which *S. cerevisiae* utilizes sucrose (3, 4, 31, 100) and that the presence of a sucrose-proton symport system may be a strain-dependent property.

In most cases, uptake of disaccharides by yeasts has been reported to occur via proton symport. Recent studies have shown that a putative facilitated maltose transport can be attributed to an experimental artifact (7). Disaccharide-proton symport systems in yeasts characteristically have a relatively high affinity constant of 2 to 6 mM (15, 32, 93, 94, 96).

The most extensively studied disaccharide uptake system in

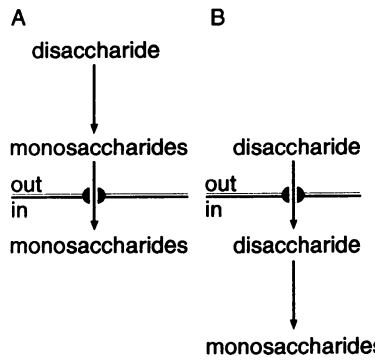

FIG. 3. Different modes of disaccharide metabolism in yeasts. (A) Extracellular hydrolysis of disaccharides followed by transport of the monosaccharides is the most common method of sucrose metabolism in yeasts. (B) Transport of disaccharides by proton-sugar symport followed by intracellular hydrolysis occurs in lactose and maltose metabolism.

yeasts is the *S. cerevisiae* maltose carrier. In this yeast, three gene products are required for maltose utilization: a maltose-specific transporter, the maltose-hydrolyzing enzyme α-glucosidase, and an activator of transcription (18, 20, 22, 67). Clusters of the three genes encoding these proteins occur in five different loci named *MAL1* to *MAL4* and *MAL6*, which exhibit high sequence homology (66). Recently, van den Broek et al. (102) compared profiles of membrane proteins in *S. cerevisiae* grown in maltose- and glucose-limited chemostat cultures. Polyacrylamide gel electrophoresis of isolated plasma membranes revealed that growth on maltose induced two membrane-associated proteins, not present in glucose-grown cells, with apparent molecular masses of 64 and 59 kDa. Partial amino acid sequencing of the 64-kDa protein revealed complete identity with amino acid sequences predicted from the DNA sequence of the *MAL61* gene, indicating that this is the inducible maltose permease of *S. cerevisiae*.

Another well-studied disaccharide transport system is the lactose carrier in *Kluyveromyces* species. This transporter, with a K_m value of about 1 mM, catalyzes lactose-proton symport (6, 32, 104). The gene coding for this transporter in *K. lactis* has been identified by transformation of *S. cerevisiae* with *LAC12*, encoding the presumed transporter, and *LAC4*, encoding β-galactosidase. The transformed *S. cerevisiae* strain could grow on lactose and, moreover, showed uncoupler-sensitive lactose transport (98). Combined with the findings that *LAC12* codes for a hydrophobic protein homologous to the *Escherichia coli* xylose-H^+ and arabinose-H^+ transporters (17), this shows that *LAC12* indeed codes for a carrier.

Regulation

Sugar uptake by yeasts is strongly regulated by environmental conditions, both at the level of enzyme synthesis and at the level of enzyme activity. When a yeast possesses multiple transport systems for a given sugar, its concentration in the environment is often a key factor in the regulation of the synthesis of the individual carriers. At high sugar concentrations, high-affinity carriers are generally repressed, but repression is relieved when the sugar concentration in the environment decreases. This type of regulation is well documented for glucose transport in, e.g., *Candida* and *Kluyveromyces* species (83, 85). A similar mechanism has been suggested for glucose transport in *S. cerevisiae* (9, 55), but in a recent study this view was questioned by suggesting that the glucose transporter(s) in this yeast might be constitutive (17). Thus, observed changes in the apparent affinity constants would involve modification of the activity of existing glucose carriers, rather than repression/derepression of carrier synthesis by switching off and on of carrier genes. Expression studies of the presumed glucose transporters, however, are not in agreement with this view, since expression of *HXT1*, *HXT2*, and *HXT3*, measured by using *lacZ* fusion proteins (51, 58) or antibodies against specific parts of the protein (119), was shown to be growth phase dependent.

A complex regulation of transport has been observed for disaccharide carriers such as those for maltose and lactose. In these cases the activity of the disaccharide transporters in a variety of yeasts (3) is governed by induction-repression mechanisms (4, 26, 76, 94).

Existing transport capacity for a sugar can also be controlled by posttranslational modification of the carrier protein. For example, in the absence of a nitrogen source for growth, the glucose transport activity in *S. cerevisiae* rapidly declines (14, 56). Inactivation in the presence of high glucose concentrations or in the absence of a nitrogen source has been observed for galactose transport (29, 62) and maltose transport (13, 44). For maltose, studies with antibodies have indicated that the disappearance of activity is presumably associated with proteolysis of the carrier (60). In addition to irreversible inactivation, maltose transport can be reversibly inactivated, depending on growth conditions (77). The physiological necessity for short-term regulation of transport activity is illustrated by studies on maltose transport with *S. cerevisiae* mutants defective in glucose repression (34, 35) and with wild-type cells exposed to a sudden change in the maltose concentration (86). Uncontrolled maltose uptake led to substrate-accelerated death, a phenomenon also known to occur in bacteria (81).

Evidently, the sugar concentration in the environment can strongly influence the kinetics of sugar uptake. As a consequence, cultivation methods used for transport studies should involve control of the sugar concentration.

CULTIVATION METHODS

With the exception of certain disaccharides, whose metabolism is initiated by extracellular hydrolysis, the specific rate of sugar uptake (v) is equal to the specific rate of sugar consumption (q_s) in growing cultures. In principle, several cultivation methods can be applied to study the kinetics of sugar uptake by growing cells. The pros and cons of these methods are briefly evaluated below.

Batch Cultivation

For studies on transport kinetics in growing cells, it is crucial to use controlled cultivation conditions. This allows manipulation of growth parameters that affect sugar transport. Shake flask cultures do not meet this requirement, since pH and dissolved-oxygen tension cannot be regulated. Both these parameters exert a strong influence on the rate of sugar transport (48, 101, 104, 108).

Batch cultivation in fermentors in which pH, dissolved-oxygen tension, and temperature are controlled may at first glance seem a suitable cultivation method for studying sugar transport in situ. The sugar concentration in the environment, although continuously decreasing (Fig. 4), can be chosen in such a way that it does not limit the rate of transport and the specific growth rate is constant ($\mu = \mu_{max}$). During exponential

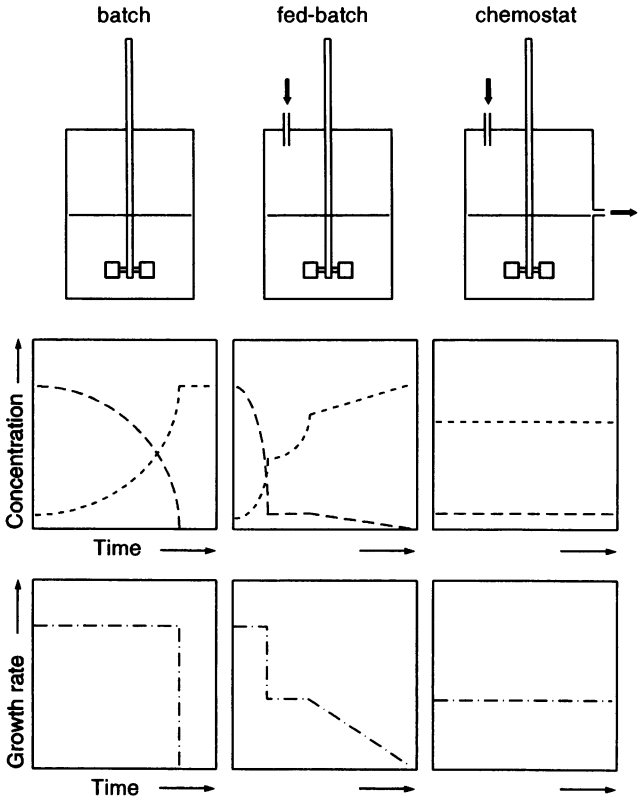

FIG. 4. Schematic representation of batch, fed-batch, and chemostat cultivation. (A) Batch cultivation. Exponential growth is observed with μ_{max} under the cultivation conditions used. After depletion of the sugar, the growth rate drops to zero. If by-products are formed, cells may enter a second growth phase during which these by-products are consumed (not shown). (B) Fed-batch cultivation. This cultivation method may start with a batch phase. After the sugar is consumed, a feed is started that allows exponential growth at a rate lower than μ_{max}. During this phase, the sugar concentration in the culture is kept constant by an exponential feed. After the limits of oxygen transfer into the reactor have been reached, the exponential feed cannot be maintained, and, as a result, the growth rate decreases. (C) Chemostat cultivation. During growth under steady-state conditions, all relevant growth parameters, including the sugar concentration in the culture, are constant in time.

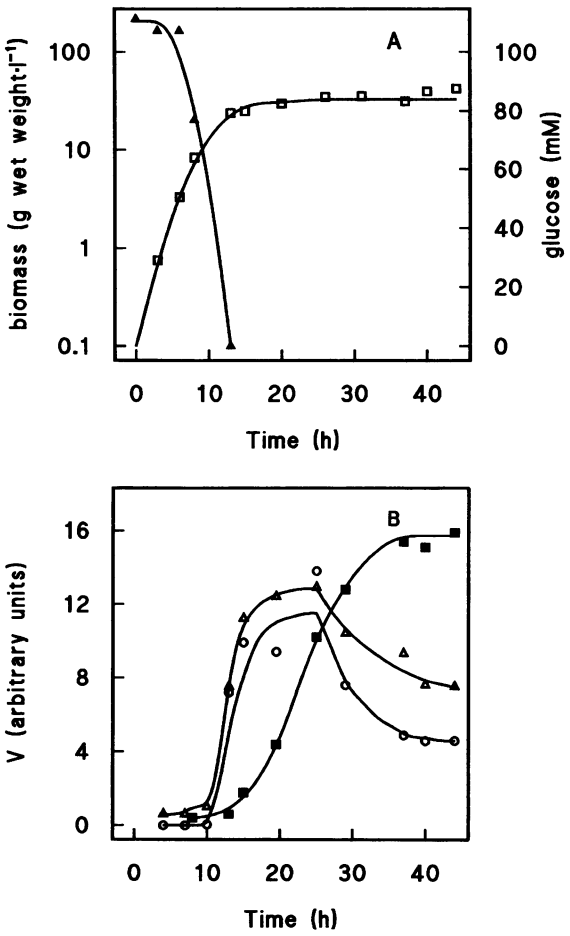

FIG. 5. Relation between sugar influx and the moment of harvesting of *K. marxianus* grown in batch culture on glucose. (A) Symbols: □, biomass production; ▲, glucose consumption. (B) Uptake velocities (in arbitrary units) of *p*-nitrophenyl-β-D-galactosidase (○), sorbose (△), and 6-deoxyglucose (■). Data from reference 26 with permission of the publisher.

growth, the specific rate of sugar consumption (q_s) is constant and is described by equation 2:

$$q_s = \mu/Y_{sx} \quad (2)$$

in which Y_{sx} is the biomass yield, expressed as amount of biomass formed per amount of sugar consumed. Only when the sugar concentration in the culture is no longer sufficient to saturate the existing transport capacity, the growth rate decreases as a result of a decrease in the rate of sugar transport. This holds only if the biomass concentration that is obtained at the end of the exponential phase is determined solely by the initial sugar concentration in the growth medium. If other nutrients (for example, the nitrogen source) are limiting, sugar transport and growth may become uncoupled as a result of changes in biomass composition and production of overflow metabolites, thus making the relation between uptake and growth much more complicated. A rapid transition to zero growth occurs upon exhaustion of the sugar. In batch cultures, the expression and properties of sugar transport systems in yeasts depend strongly on the time of harvesting (12, 26) (Fig. 5).

The situation depicted in Fig. 4 with respect to the kinetics of growth and sugar consumption is more complicated when by-products that affect cellular physiology are formed. This occurs, for example, in aerobic batch cultures of *S. cerevisiae* and other Crabtree-positive yeasts, which exhibit alcoholic fermentation in the presence of sugar concentrations above 1 mM (115). Apart from ethanol, other products that negatively affect biomass yield, such as acetic acid, may be formed.

Fed-Batch Cultivation

An intrinsic disadvantage of batch cultivation is that the data it provides on sugar uptake can be extrapolated only to situations in which sugar is present in excess ("in excess" is used here to indicate concentrations that do not limit the growth rate). Such studies have little relevance for the industrial cultivation of yeasts in sugar-limited fed-batch cultures (Fig. 4). In the latter system, often started with a batch phase, sugar is supplied to the culture at a growth-limiting rate (i.e., the growth rate that is allowed by the rate of sugar addition is lower than μ_{max}).

In fed-batch systems, a constant specific growth rate can be accomplished by an exponentially increasing feed rate that

takes into account the increase in culture volume and biomass concentration. In practice, fed-batch cultivations are not operated at a constant growth rate but involve a feed profile that leads to a continuous decrease in growth rate. This profile is governed by a variety of factors, including the oxygen transfer and cooling capacities of industrial bioreactors (8, 27). In addition to its experimental complexity, the use of fed-batch cultivation for fundamental studies on sugar transport suffers from the potential danger of a change in growth conditions related to the increase in biomass. For example, the formation of many toxic byproducts is linearly proportional to the amount of biomass, whereas adverse effects may be exponentially related to by-product concentration. Even in the absence of by-product formation, growth conditions that result in a constant (submaximal) growth rate can be maintained for only a relatively short period (hours rather than days). After this period, accumulation of biomass leads to a situation in which oxygen transfer properties of the fermentor set the limits for the medium feed.

Chemostat Cultivation

Chemostat cultivation does not suffer from the disadvantages of batch and fed-batch cultivation and is therefore better suited for transport studies. In a chemostat, nutrients are continuously fed to the culture. The culture volume is kept constant by continuous removal, at the same rate, of culture fluid containing biomass, products, and nondepleted nutrients (Fig. 4). The medium that is fed into the culture is designed in such a way that one nutrient of choice (for example, the sugar) determines the biomass concentration in the culture. As a result, this limiting nutrient is almost completely consumed and its residual concentration in the culture is very low. The biomass yield (Y_{sx}) on the limiting nutrient is given by equation 3, in which C_{si} is the reservoir concentration of the limiting nutrient and C_s and C_x are the residual substrate concentration and the biomass concentration in the culture, respectively:

$$Y_{sx} = C_x/(C_{si} - C_s) \quad (3)$$

The flow rate at which the medium is fed to the culture (liters per hour) divided by the volume of the culture (liters) equals the dilution rate, D (reciprocal hours) (equation 4):

$$D = \frac{\text{flow rate}}{\text{culture volume}} \quad (4)$$

The reciprocal value of the dilution rate equals the time required for one volume change. Usually, approximately five volume changes after a change in the growth conditions, a steady-state situation is reached, in which the growth rate μ equals the dilution rate D, according to equation 5:

$$\mu = D = \frac{\mu_{max} C_s}{K_s + C_s} \quad (5)$$

in which C_s is the residual concentration of the growth-limiting nutrient and K_s is the affinity constant for growth on this nutrient (64). In this steady state, the concentration of all nutrients, including the growth-limiting substrate, is constant over time.

As a result of the constant growth conditions, the physiology of the microorganism also remains constant. This includes the sugar carrier content of the cells and the kinetics of sugar transport, which are not dependent on the time of harvesting. Therefore, steady-state chemostat cultures are ideally suited as a reproducible source of cell suspensions for sugar transport studies.

The use of chemostat cultivation is particularly advantageous in transport studies with *S. cerevisiae*. Aerobic batch cultivation of this yeast on sugars leads to a characteristic pattern. In the first phase, the sugar is fermented to alcohol, as a result of repression of various respiratory enzymes. When the sugar is exhausted, a lag phase is observed, during which the yeast adapts to a second growth phase on ethanol. Many differences reported in the literature with respect to properties of sugar transport in *S. cerevisiae* can probably be ascribed to the physiological status of the yeast, which is strongly dependent on the phase of growth and, consequently, on the time of harvesting.

Chemostat cultivation allows evaluation of the effects of specific environmental parameters on sugar transport in growing cultures. For example, the effect of growth temperature can be studied independently of the effects of growth rate. In batch cultures, this is not possible, since a change in growth temperature will also result in a change in growth rate. As a result, it is not possible to conclude whether observed differences in sugar transport are due to a change in temperature, a change in growth rate, or both.

The general principles of chemostat cultivation and the methods used have been amply reviewed (47, 80). Below, some aspects that are of particular relevance for sugar uptake studies are briefly discussed.

METHODS USED FOR CHEMOSTAT CULTIVATION IN RELATION TO SUGAR TRANSPORT STUDIES

Medium Composition

Since the uptake of solutes by growing cultures is strongly dependent on environmental conditions, these conditions must be constant and defined. The basis for reliable chemostat cultivation, i.e., for defined growth conditions, is an appropriate design of the growth medium. In sugar-limited chemostat cultures, all inorganic nutrients and vitamins must be present in excess. Optimization of growth media with respect to the major nutrients is facilitated by the use of macroscopic balances. For example, formation of *S. cerevisiae* biomass in aerobic, glucose-limited chemostat cultures grown at a dilution rate of 0.10 h^{-1} can be described by equation 6 (114):

$$1.089C_6H_{12}O_6 + 0.540NH_4^+ + 2.597O_2 \rightarrow$$
$$1.000C_{3.75}H_{6.6}O_{2.18}N_{0.54}(100\text{ g of biomass}) \quad (6)$$
$$+ 2.786CO_2 + 0.540H^+ + 4.044H_2O$$

From this equation, it is clear that to obtain a biomass concentration of 5 g $liter^{-1}$, at least (5/100) × 0.54 = 27 × 10^{-3} mol of ammonium $liter^{-1}$ must be present in the growth medium. If this concentration of ammonium is not present, growth will no longer be limited by the sugar supply and the culture will be nitrogen limited. This may result in the accumulation of sugar in the culture. Under such conditions, yeasts generally do not express the high-affinity uptake systems that are present in sugar-limited cultures (85).

In addition to the major nutrients, micronutrients (trace elements and vitamins) must be present in excess. In anaerobic cultures, the vitamin mixture should include ergosterol and unsaturated fatty acids (1, 2). A shortage in the supply of an essential nutrient can be detected by increasing C_{si}; limitation by a compound other than the sugar will result in different values of C_s. Only if C_s is independent of C_{si} and the biomass

concentration in the culture is linearly proportional to the reservoir concentration of the growth-limiting nutrient (C_{si}) can chemostat cultures be assumed to follow Monod kinetics.

By using balanced mineral media and adequate fermentation equipment, constant conditions (dissolved-oxygen tension, temperature, pH, biomass concentration, dilution rate, etc.) are relatively easy to achieve. Detailed discussions on foam control, the choice of appropriate equipment, methods for dissolved-oxygen control, etc., are beyond the scope of this paper.

Determination of Residual Sugar Concentration

A parameter of obvious importance for studies on sugar transport in chemostat cultures is C_s, the residual sugar concentration in the culture. In comparison with studies on the kinetics of intracellular enzymes, studies of the relation between C_s and in situ carrier activity appear to be relatively straightforward. For measurement of C_s, sample preparation encompasses only the separation of culture supernatant from the cells; permeabilization of cells is not required. However, in sugar-limited chemostat cultures, and in particular those of Crabtree-negative yeasts, the residual sugar concentration is generally very low (usually well below 1 mM [Table 1]). Therefore, rapid sampling and separation of biomass from the growth medium are required to prevent consumption of the sugar during sampling. The following example may serve to illustrate this.

In an aerobic, glucose-limited chemostat culture of *Candida utilis* growing at $D = 0.3\ h^{-1}$ with a reservoir concentration of 5 g of glucose liter^{-1}, the biomass yield and residual glucose concentration are 0.5 g of biomass g of glucose^{-1} and 18 μM, respectively (83). From these data, it follows that the specific rate of glucose consumption (q_s [see equation 2]) is 3.33 mmol of glucose g of biomass^{-1} h^{-1}. Since the reservoir concentration is 5 g liter^{-1} and $Y_{sx} = 0.5$ g of biomass g of glucose^{-1}, the volumetric rate of sugar consumption equals $0.5 \times 5 \times 3.33 = 8.33$ mmol liter^{-1} h^{-1} = 2.3 μM s^{-1}. If this rate of sugar consumption were to continue during sampling, a sampling time of 8 s would result in complete depletion of the sugar initially present in the sample.

In practice, sampling times of 1 to 3 s can be obtained by filtering culture samples through a cellulose-acetate filter (33). An alternative method is to directly transfer a culture sample into liquid nitrogen (83, 84). After the sample has thawed on ice, the cell-free supernatant can be recovered by centrifugation or filtration at 0°C. Yet another option is sampling by inclusion of a dialysis probe in the fermentor (85). It is obvious that for measurements of residual substrate concentrations in chemostat cultures, low cell densities are preferred, since this will reduce substrate consumption during sampling. This approach has been followed in the elegant studies by Egli et al. (33) on the growth kinetics of *E. coli*, in which residual substrate concentrations were in the nanomolar range. It is important to stress that the affinity for the growth-limiting substrate imposes a lower limit on the steady-state biomass concentrations that can be used in chemostat cultures; equation 5 is valid only when the reservoir concentration of the growth-limiting substrate (C_{si}) is at least 2 orders of magnitude higher than the residual substrate concentration (C_s) (80).

Whatever the sampling method, it should be validated by measuring C_s at different values of C_{si}. C_{si} determines the biomass concentration in the culture (equation 3), but it does not affect C_s, which depends only on μ, μ_{max}, and K_s (equation 5). If different values for C_s are observed when C_{si} is varied, this may be indicative of substrate consumption during sampling and of the method being inappropriate. Alternatively, it may point to limitation by a nutrient other than the sugar substrate.

Using the liquid-nitrogen sampling technique, Postma et al. (83) observed the same C_s at different values of C_{si} in glucose-limited chemostat cultures of *C. utilis*. Furthermore, calculation of μ_{max} from equation 5, using C_s measurements at different growth rates, gave the same value for this parameter as observed in batch cultures.

For determination of the in situ kinetics of sugar consumption in chemostat cultures, a number of prerequisites must be fulfilled. The fermentor must have excellent mixing properties to avoid sugar gradients. Especially at low dilution rates, care must be taken to avoid discontinuous, dropwise substrate addition as a result of low pump rates. This problem can be avoided by using large culture volumes. The mathematics of chemostat cultivation, as briefly described in equations 3 and 5, apply only if the biomass concentration in the culture is identical to the biomass concentration in the culture effluent. In particular, when effluent is removed continuously from the culture surface, selective removal of either biomass or extracellular medium may occur (70). This phenomenon can be avoided by effluent removal from below the surface of the culture. The culture volume may then be kept constant, for example, by coupling the effluent pump to a surface contact sensor.

Wall growth can cause major problems in chemostat experiments. In particular, at low biomass concentrations even barely visible growth on the walls of fermentor vessels may make a quantitative contribution to the overall rate of sugar consumption. Wall growth may thus lead to an underestimation of residual substrate concentrations.

A special problem may be encountered with *S. cerevisiae* strains. In respiratory, sugar-limited cultures of this yeast, spontaneous synchronization of the cell cycle may occur (75, 116), which is associated with oscillations in sugar transport and metabolism. In some strains these oscillations do not damp out. When alternatives are available, yeast strains with a strong tendency toward wall growth or oscillations should not be chosen as model organisms for chemostat studies.

MANIPULATION OF METABOLIC FLUXES IN CHEMOSTAT CULTURES

Since, in substrate-limited chemostat cultures, the dilution rate equals the specific growth rate, equation 2 can be rewritten as

$$q_s = D/Y_{sx} \quad (7)$$

This implies that in chemostat cultures, the specific rate of substrate consumption can be varied either by manipulation of the growth rate or by controlled modification of the biomass yield. A third option to obtain a controlled variation of metabolic fluxes is growth on mixed substrates. Each of these three possibilities will be briefly discussed below.

Variation of Dilution Rate

In many yeasts, the biomass yield from the substrate is virtually constant over a wide range of dilution rates (80). In these cases, equation 7 predicts a linear relationship between the dilution rate and the specific rate of substrate consumption. This implies that the rate of substrate consumption can be manipulated by varying the dilution rate. Indeed, a linear relationship between growth rate and q_s was observed when the dilution rate of aerobic, sugar-limited chemostat cultures

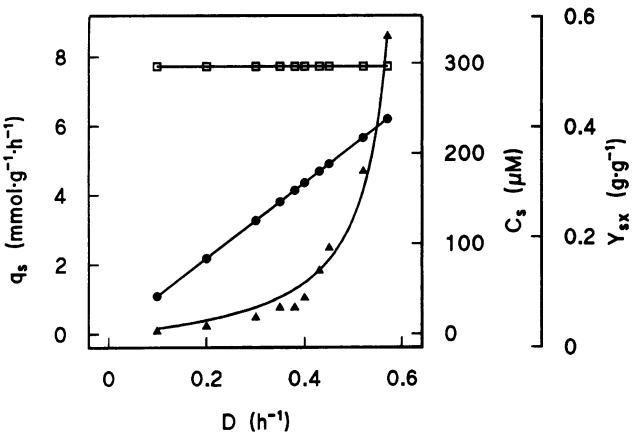

FIG. 6. Residual glucose concentration (C_s [▲]), specific glucose uptake rate (q_s [●]), and biomass yield (Y_{sx} [□]) as a function of the dilution rate in aerobic glucose-limited chemostat cultures of *C. utilis* CBS 621. The glucose concentration in the cultures was determined after rapid sampling in liquid nitrogen. Data from reference 83.

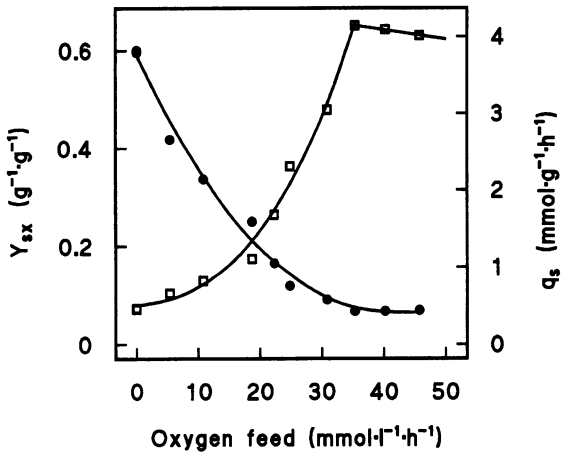

FIG. 7. Biomass yield (□) and specific maltose uptake rate (●) as a function of the oxygen feed in maltose-limited chemostat cultures of *S. cerevisiae* CBS 8066. The experiments were performed at a fixed dilution rate of 0.10 h^{-1}. Data from reference 123.

of *C. utilis* cultures was varied between 0.10 and 0.50 h^{-1} (Fig. 6).

It should be stressed that the simple linear relationship between D and q_s applies only when the biomass yield does not change with the dilution rate. At very low dilution rates, energy requirements for maintenance processes affect the biomass yield (80), which therefore cannot be considered a constant. This will make the relation between D and q_s at low growth rates more complex. As discussed below, changes in sugar metabolism that occur at high growth rates in some yeasts may also complicate the relationship between μ and q_s.

Variation of Biomass Yield

Variation of the metabolic flux by manipulating the dilution rate is inevitably associated with changes in growth rate. According to equations 2 and 7, q_s can also be manipulated independently of the growth rate by modifying the biomass yield on the growth-limiting substrate. In yeasts, this can be achieved in various ways.

In facultatively fermentative yeasts, the biomass yield of chemostat cultures that respire sugar is much higher than the yield of fermentative cultures. For example, in aerobic, glucose-limited chemostat cultures of *S. cerevisiae* grown at D = 0.10 h^{-1}, Y_{sx} = 0.5 g g^{-1}. In anaerobic cultures grown at the same dilution rate, Y_{sx} = 0.1 g of biomass (g of glucose)$^{-1}$, which is associated with a fivefold-higher glucose uptake rate. This implies that q_s can be varied by controlled manipulation of the oxygen feed rate to sugar-limited chemostat cultures (Fig. 7) (122).

A rather complex modulation of sugar transport rates, caused by simultaneous changes of μ and Y_{sx}, occurs in aerobic, glucose-limited chemostat cultures of *S. cerevisiae*. At low dilution rates, growth of this yeast is fully respiratory and the glucose uptake rate increases linearly with increasing D (36, 50, 84). However, above a certain critical dilution rate, respiration and alcoholic fermentation occur simultaneously, resulting in a decrease of Y_{sx}. Above the critical dilution rate, the simultaneous increase of D and decrease of Y_{sx} result in a strongly enhanced rate of sugar uptake (Fig. 8).

An alternative experimental approach to enhance the sugar transport rate at a fixed dilution rate is the addition of nonmetabolizable weak organic acids (e.g., benzoic acid) to the growth medium of sugar-limited chemostat cultures (112, 113). These compounds dissipate the transmembrane pH gradient by diffusing from the acidic extracellular environment into the near-neutral cytosol. Inside the cells, the acid molecules dissociate. To prevent acidification of the cytoplasm, the released protons must be expelled from the cell by the plasma membrane ATPase complex. As a result of the enhanced ATP requirement for intracellular pH homeostasis, less ATP is available for biosynthetic purposes. This in turn results in a decrease of the biomass yield (Y_{sx}) and, consequently, in an increase of the sugar uptake rate (Fig. 9).

Growth on Mixed Substrates

In batch cultures, the utilization of substrate mixtures by yeasts often occurs via a sequential (diauxic) pattern (64) because of catabolite repression phenomena. In contrast, the low residual-substrate concentrations in sugar-limited chemo-

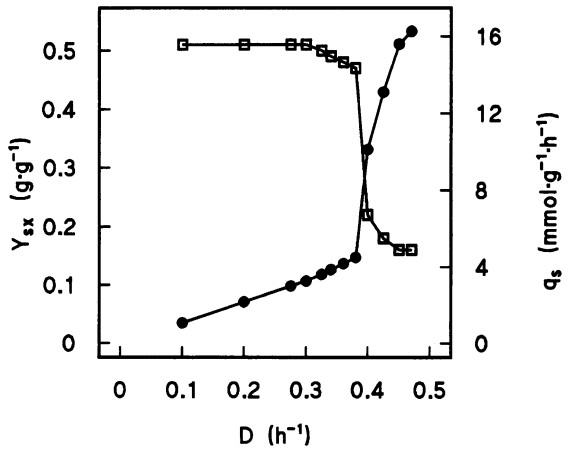

FIG. 8. Physiology of *S. cerevisiae* CBS 8066 as a function of growth rate in aerobic glucose-limited chemostat cultures. Above a dilution rate of 0.38 h^{-1}, alcoholic fermentation sets in. As a result, the biomass yield (Y_{sx} [□]) decreases. Since growth remains sugar limited, a disproportionate increase in the specific rate of glucose consumption (q_s [●]) occurs. Data from reference 87.

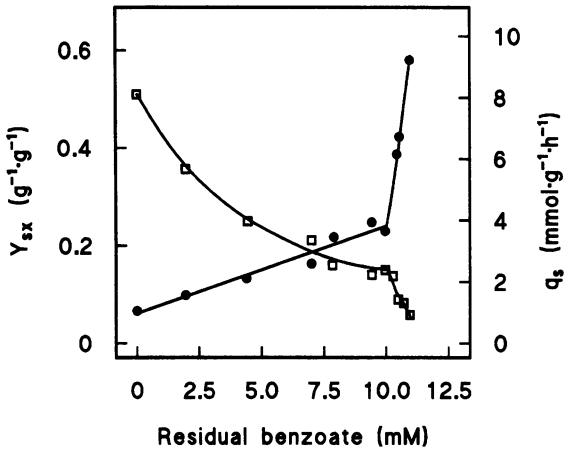

FIG. 9. Biomass yield (□) and specific glucose uptake rate (●) as a function of benzoate concentration during glucose-limited cultivation of *S. cerevisiae* CBS 8066 in aerobic chemostat cultures. The experiments were performed at a fixed dilution rate of 0.10 h^{-1}. Data from reference 113 with permission of the publisher.

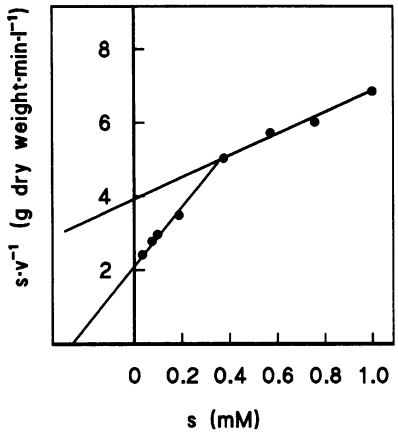

FIG. 11. Hanes plot of the kinetics of [^{14}C]glucose transport by cells of *C. utilis* CBS 621 grown in an aerobic, glucose-limited chemostat culture at a dilution rate of 0.52 h^{-1}, indicating the presence of two kinetically distinct components. Data from reference 83.

stat cultures allow the simultaneous utilization of sugar mixtures or mixtures of sugars and other substrates (33, 45). Thus, cultivation on mixed substrates offers yet another possibility to manipulate the specific rate of sugar transport in chemostat cultures. For example, glucose and ethanol can be utilized simultaneously in dual-substrate-limited chemostat cultures of *S. cerevisiae* (28, 42). In such cultures, the specific rates of glucose and ethanol consumption can be manipulated not only by varying the dilution rate but also by changing the relative concentrations of glucose and ethanol in the medium feed (Fig. 10).

REGULATION OF SUGAR TRANSPORT IN CHEMOSTAT CULTURES

As discussed above, chemostat cultivation offers unique possibilities to manipulate the rate of sugar transport in growing cultures. So far, however, little is known about the mechanisms used by yeast cells to adapt their transport kinetics to accommodate these variations in flux. One example, transport of glucose in glucose-limited chemostat cultures of *C. utilis*, is discussed below.

In most yeasts, more than one system for uptake of glucose may be present during growth on this sugar. In such cases, the specific rate of sugar consumption (q_s) is equal to the sum of the transport rates (v) by the individual carriers, analogous to equation 1. The kinetic constants K_m and V_{max} for the individual carriers can be determined by measuring initial rates of [^{14}C]glucose uptake by culture samples at different substrate concentrations. In such cases, nonlinear kinetic plots can be obtained (Fig. 11). When the residual glucose concentration in the culture is also known, the in situ contribution of the

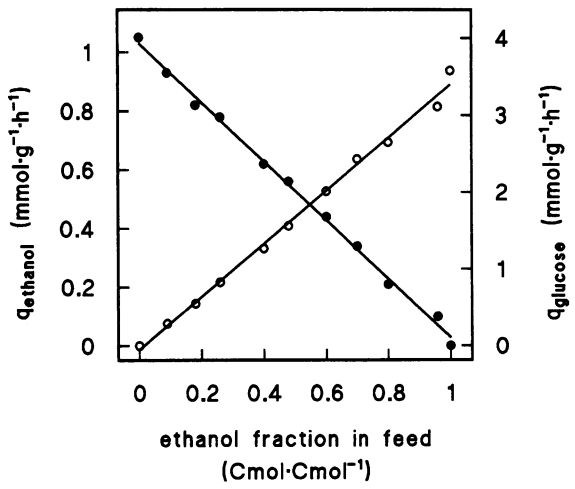

FIG. 10. Specific rates of ethanol (○) and glucose (●) consumption in dual-substrate-limited chemostat cultures of *S. cerevisiae* YLD01 grown on mixtures of glucose and ethanol. The fraction of ethanol in the reservoir medium is expressed as the fraction of substrate carbon that is present as ethanol. The experiments were performed at a fixed dilution rate of 0.10 h^{-1} (28).

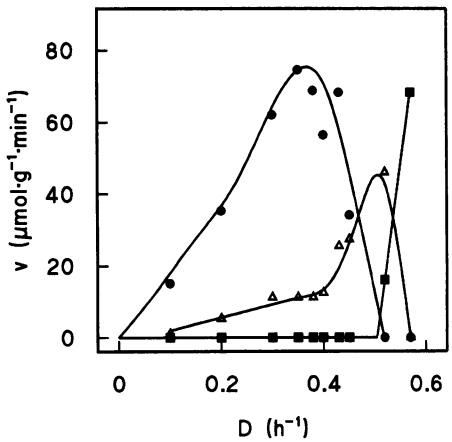

FIG. 12. Contribution of three kinetically distinct uptake systems to glucose uptake in aerobic, glucose-limited chemostat cultures of *C. utilis* CBS 621. The uptake rate via each of the carriers was calculated from the residual glucose concentration in the cultures and the kinetic constants of the carriers as determined by uptake rates of [^{14}C]glucose at different concentrations (see Fig. 6). The three carriers differ by approximately 1 order of magnitude in their K_m for glucose: ●, 25 μM; △, 190 μM; ■, 2,000 μM. Data from reference 83.

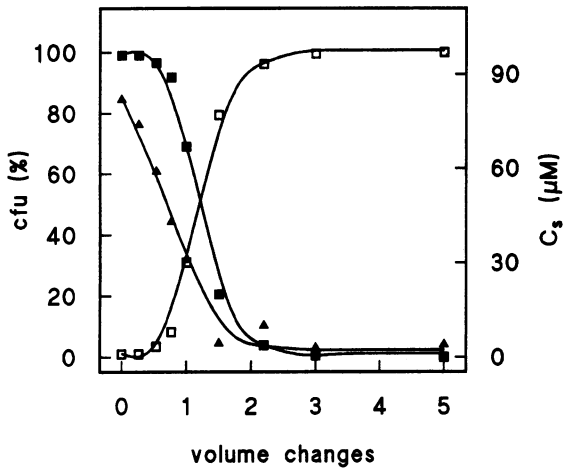

FIG. 13. Competition between *S. cerevisiae* CBS 8066 (■) and *C. utilis* CBS 621 (□) in an aerobic, glucose-limited chemostat culture at a dilution rate of 0.10 h^{-1}. At zero time, a pure culture of *S. cerevisiae* was inoculated with 1% (on a dry-weight basis) of *C. utilis* cells. The numbers of CFU of both yeasts are expressed as the percentage of the total CFU. Numbers are plotted as a function of the number of volume changes. The residual glucose concentration (▲) decreases during the competition to the value that is typical for pure cultures of *C. utilis*. *S. cerevisiae* cannot maintain itself in the mixed culture because of its lower affinity for glucose. Data from reference 82.

different carriers can be calculated (Fig. 12). From Fig. 12 it is clear that in glucose-limited chemostat cultures of *C. utilis*, the contribution of the different carriers varies with the dilution rate. At dilution rates approaching μ_{max} (0.59 h^{-1}), synthesis of the two high-affinity carriers is repressed and a low-affinity carrier plays a predominant role.

The above results illustrate the usefulness of chemostat cultivation: in batch cultures, growth occurs at μ_{max} and the high-affinity systems are not detectable. Only during the very short transition phase between the exponential growth phase and the stationary phase is synthesis of these carriers derepressed. Studies on the mechanism and kinetics of high-affinity sugar transport in yeasts are therefore generally performed with substrate-deprived cells or with cells grown on substrates on which synthesis of high-affinity carriers is derepressed.

In general, the facilitated-diffusion glucose transport systems of Crabtree-positive yeasts, such as *S. cerevisiae* and *Schizosaccharomyces pombe*, have a much higher K_m for glucose than do the high-affinity proton symport mechanisms that are common in Crabtree-negative yeasts (Table 1). The observed correlation between transport kinetics and residual-sugar concentrations in glucose-limited chemostat cultures (Table 1) suggests that the kinetic properties of glucose uptake may to a large extent determine the growth kinetics. The importance of transport kinetics for growth of yeasts under sugar-limited conditions becomes particularly evident when different yeasts compete for a single growth-limiting sugar. As can be predicted from Table 1, *S. cerevisiae* is rapidly outcompeted in mixed cultures by the Crabtree-negative yeast *C. utilis* during aerobic, glucose-limited growth (Fig. 13). The better adaptation of various Crabtree-negative yeasts to growth at low sugar concentrations offers an explanation for the competitive advantage of so-called wild yeasts when these contaminate industrial baker's yeast production processes (82).

CHEMOSTAT CULTIVATION AND ENERGETICS OF SUGAR TRANSPORT

The amount of ATP required for sugar-proton symport is determined by two stoichiometries: the sugar-proton stoichiometry of the proton symport carrier and the ATP-proton stoichiometry of the plasma membrane ATPase complex (Fig. 1). In principle, the overall ATP requirement for sugar uptake can be calculated by independent determination of each of these two stoichiometries, for example from in vitro experiments.

The energy required for proton symport reduces the amount of energy available for biomass formation and can therefore be expected to cause a decrease in the biomass yield. Measurement of this decrease can be used for in vivo determination of the ATP requirement for sugar-proton symport. A prerequisite is that the transport step consumes a significant fraction of the ATP equivalents that are formed in the dissimilation of the sugar. This is the case during fermentative growth, when substrate-level phosphorylation yields only 2 mol of ATP per mol of hexose sugar. Accurate determination of biomass yields furthermore requires that all culture conditions that influence growth efficiency be controlled. Therefore, chemostat cultivation is the only cultivation method applicable for this type of in vivo investigation.

The energy requirement for maltose-proton symport in *S. cerevisiae* has been studied by comparison with the facilitated diffusion of glucose in anaerobic sugar-limited chemostat cultures (121). *S. cerevisiae* was grown on both sugars under identical cultivation conditions. The biomass yield, expressed as the amount of biomass formed per amount of consumed hexose units, was 25% lower during growth on maltose than during growth on glucose. Apparently, 25% of the four ATP molecules formed during maltose fermentation (i.e., one ATP molecule per maltose molecule) was needed for uptake of this disaccharide. This increased rate of substrate-level phosphorylation has to be sustained by an increased rate of glycolysis. Indeed, specific ethanol and carbon dioxide production rates were substantially higher during growth on maltose than during growth on glucose (121).

To confirm this conclusion, *S. cerevisiae* was grown on mixtures of glucose and maltose, which were fed simultaneously to the sugar-limited chemostat cultures. The biomass yield and ethanol and carbon dioxide production rates varied with increasing maltose-to-glucose ratios in the reservoir media as predicted by a metabolic model, which was based on the assumption that one ATP equivalent is required for maltose uptake (Fig. 14).

The maltose/ATP stoichiometry of 1 determined in vivo is in accordance with a maltose/proton stoichiometry for the maltose/proton symporter of 1 (96, 108) and a proton/ATP stoichiometry of the plasma membrane ATPase of 1 (61, 68, 78) as determined in in vitro experiments.

FUTURE PROSPECTS

A number of genes involved in sugar transport have been cloned from yeasts and sequenced (Table 2). The predicted gene products are highly hydrophobic proteins, which share homology with bacterial and mammalian sugar transporters. All the putative gene products have, potentially, 9 to 12 membrane-spanning domains, and many of them contain potential N-linked glycosylation sites and recognition sites for cyclic AMP (cAMP)-dependent protein kinase. The latter observation suggests that cAMP may be involved in the regulation of sugar transport capacity in yeasts.

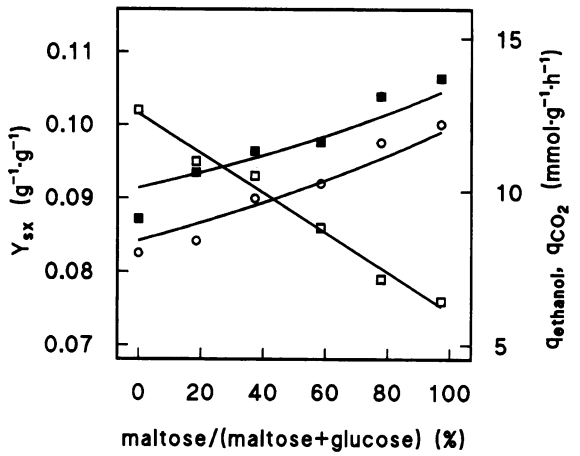

FIG. 14. Specific rates of ethanol (○) and carbon dioxide (■) production and biomass yield (□) in anaerobic, sugar-limited chemostat cultures of *S. cerevisiae* CBS 8066 grown at a fixed dilution rate of 0.10 h^{-1} on mixtures of glucose and maltose. Specific production rates (q) were calculated from the biomass concentration in the culture and the amount of ethanol and carbon dioxide produced. Experimental data are plotted as a function of the percentage of the total hexose consumption that was consumed as maltose. The curves for ethanol and carbon dioxide production are slightly bent because the biomass yield on maltose is 25% lower than on glucose (see the text). Biomass composition (C, H, N, S, and protein) and glycerol production did not change as a function of the medium composition. Data from reference 121.

In many cases, the proposition that DNA sequences represent a structural gene for a sugar transport protein is based on circumstantial evidence. For example, it has not been unequivocally proven that any of the genes listed in Table 2 code for proteins that catalyze the translocation of sugars over the plasma membrane. The most convincing data on the direct involvement of any of these genes in sugar transport have been obtained with *LAC12* from *Kluyveromyces lactis*. After integration in *S. cerevisiae*, this yeast acquired the capacity to transport lactose (98). It is still conceivable that the other genes listed in Table 2 encode a regulatory protein involved in sugar transport, rather than the structural gene for a sugar transport protein. Future work, involving the reconstitution of purified gene products in plasma membrane vesicles, is required to reveal the exact physiological function of these gene products.

Chemostat cultivation can be an important tool for further studies on the molecular biology of sugar transport. Under the controlled growth conditions of the chemostat, yeasts can be induced to synthesize maximal amounts of a particular carrier, thus facilitating its isolation and characterization. The potential uses of this approach are illustrated by recent work on the *MAL61* gene product of *S. cerevisiae*, which, after growth in maltose-limited chemostat cultures, could be directly observed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis of plasma membrane preparations (102).

In this review we have attempted to illustrate the usefulness of chemostat cultivation in studies on the mechanism and kinetics of sugar transport by yeasts in relation to environmental conditions. To understand the role of the various genes that have been implicated in sugar uptake in yeasts, it is of key importance to study their differential expression as a function of growth conditions (e.g., sugar concentration, pH, and growth rate). Quantitative determination of mRNA and protein levels in chemostat cultures grown under defined conditions may be a valuable technique for this type of studies.

The rapidly expanding body of knowledge about the molecular genetics of sugar transport in yeasts offers a number of intriguing possibilities. Theoretically, the genetic modification mode of sugar transport can be used to increase the ethanol yield on sugar substrates, which may be relevant for large-scale ethanol production (121). Comparative chemostat studies of wild-type and mutant strains with different levels of expression of sugar uptake systems may be used to check the hypothesis that sugar transport is a major rate-determining step in sugar metabolism in yeasts, a problem that is interesting not only from an academic point of view. For example, overexpression of the maltose carrier gene in *S. cerevisiae* resulted in a strain with enhanced ability to leaven maltose-containing doughs (72, 73). Whether and to what extent other sugar carriers similarly exert a high degree of control over the glycolytic flux must await the identification of these carriers and the molecular cloning of the responsible genes.

ACKNOWLEDGMENTS

We thank Karel van Dam and Mike Walsh for making available unpublished results.

This work was supported by Biotechnological Sciences Delft-Leiden.

REFERENCES

1. **Andreasen, A. A., and T. J. B. Stier.** 1953. Anaerobic nutrition of *Saccharomyces cerevisiae*. I. Ergosterol requirement for growth in a defined medium. J. Cell. Comp. Physiol. **41**:23–36.
2. **Andreasen, A. A., and T. J. B. Stier.** 1954. Anaerobic nutrition of *Saccharomyces cerevisiae*. II. Unsaturated fatty acid requirement for growth in a defined medium. J. Cell. Comp. Physiol. **43**:271–281.
3. **Barnett, J. A.** 1976. The utilization of sugars by yeasts. Adv. Carbohydr. Chem. Biochem. **32**:125–234.
4. **Barnett, J. A.** 1981. The utilization of disaccharides and some other sugars by yeasts. Adv. Carbohydr. Chem. Biochem. **39**:347–404.
5. **Barnett, J. A., R. W. Payne, and D. Yarrow.** 1983. Yeast: characteristics and identification. Cambridge University Press, London.
6. **Barnett, J. A., and A. P. Sims.** 1982. The requirement of oxygen for the active transport of sugars into yeasts. J. Gen. Microbiol. **128**:2303–2312.
7. **Benito, B., and R. Lagunas.** 1992. The low-affinity component of *Saccharomyces cerevisiae* maltose transport is an artifact. J. Bacteriol. **174**:3065–3069.
8. **Beudeker, R. F., H. W. van Dam, J. B. van der Plaat, and K. Vellenga.** 1990. Developments in baker's yeast production, p. 103–146. *In* H. Verachtert and R. De Mot (ed.), Yeast biotechnology and biocatalysis. Marcel Dekker, Inc., New York.
9. **Bisson, L. F.** 1988. High-affinity glucose transport in *Saccharomyces cerevisiae* is under general glucose repression control. J. Bacteriol. **170**:4838–4845.
10. **Bisson, L. F., and D. F. Fraenkel.** 1983. Involvement of kinases in glucose and fructose uptake by *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **80**:1730–1734.
11. **Bisson, L. F., and D. F. Fraenkel.** 1983. Transport of 6-deoxyglucose in *Saccharomyces cerevisiae*. J. Bacteriol. **155**:995–1000.
12. **Bisson, L. F., and D. F. Fraenkel.** 1984. Expression of kinase-dependent glucose uptake in *Saccharomyces cerevisiae*. J. Bacteriol. **159**:1013–1017.
13. **Busturia, A., and R. Lagunas.** 1985. Identification of two forms of the maltose transport system in *Saccharomyces cerevisiae* and their regulation by catabolite inactivation. Biochim. Biophys. Acta **820**:324–326.
14. **Busturia, A., and R. Lagunas.** 1986. Catabolite inactivation of the glucose transport system in *Saccharomyces cerevisiae*. J. Gen. Microbiol. **132**:379–385.
15. **Carvalho-Silva, M., and I. Spencer-Martins.** 1990. Modes of

lactose uptake in the yeast species *Kluyveromyces marxianus*. Antonie van Leeuwenhoek Int. J. Gen. Mol. Microbiol. **57:**77–81.
16. **Celenza, J. L., L. Marshall-Carlson, and M. Carlson.** 1988. The yeast *SNF3* gene encodes a glucose transporter homologous to the mammalian protein. Proc. Natl. Acad. Sci. USA **85:**2130–2134.
17. **Chang, Y. D., and R. C. Dickson.** 1988. Primary structure of the lactose permease gene from the yeast *Kluyveromyces lactis*. Presence of an unusual transcript structure. J. Biol. Chem. **263:**16696–16703.
18. **Charron, M. J. R., A. Dubin, and C. A. Michels.** 1986. Structural and functional analysis of the *MAL1* locus of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **6:**3891–3899.
19. **Cheng, Q., and C. A. Michels.** 1989. The maltose permease encoded by the *MAL61* gene of *Saccharomyces cerevisiae* exhibits both sequence and structural homology to other sugar transporters. Genetics **123:**477–484.
20. **Cheng, Q., and C. A. Michels.** 1991. *MAL11* and *MAL61* encode the inducible high-affinity maltose transporters of *Saccharomyces cerevisiae*. J. Bacteriol. **173:**1817–1820.
21. **Clifton, D., R. B. Walsh, and D. G. Fraenkel.** 1993. Functional studies of yeast glucokinase. J. Bacteriol. **175:**3289–3294.
22. **Cohen, J. D., M. J. Goldenthal, T. Chow, B. Buchferer, and J. Marmur.** 1985. Organization of the *MAL* loci in *Saccharomyces cerevisiae*. Physical identification and functional characterization of three genes of the *MAL6* locus. Mol. Gen. Genet. **200:**1–8.
23. **Coons, D. M., D. Wendell, and L. F. Bisson.** 1993. High-affinity glucose uptake in *Saccharomyces cerevisiae*, p. 10–11. *In* M. C. Loureiro-Dias (ed.), Proceedings of the 11th Small Meeting on Yeast Transport and Energetics. Vale de Lobos, Portugal. Pasmans, The Hague, The Netherlands.
24. **Cortassa, S., and M. A. Aon.** 1993. Sugar uptake controls catabolism in yeast. Metabolic control analysis of glycolysis and the branch to Krebs cycle and fermentation in *Saccharomyces cerevisiae*, p. 116–118. *In* W. A. Scheffers and J. P. van Dijken (ed.) Proceedings of the 16th International Specialized Symposium on Yeasts. Arnhem, The Netherlands. Pasmans, The Hague, The Netherlands.
25. **Deák, T.** 1978. On the existence of H^+-symport in yeasts. A comparative study. Arch. Microbiol. **1978:**2005–2011.
26. **De Bruijne, A. W., J. Schuddemat, P. J. A. van den Broek, and J. van Steveninck.** 1988. Regulation of sugar transport systems of *Kluyveromyces marxianus*: the role of carbohydrates and their catabolism. Biochim. Biophys. Acta **939:**569–576.
27. **De Hollander, J. A.** 1993. Kinetics of microbial product formation and its consequences for the optimization of fermentation processes. Antonie van Leeuwenhoek Int. J. Gen. Mol. Microbiol. **63:**375–381.
28. **De Jong-Gubbels, P., J. P. van Dijken, and J. T. Pronk.** Unpublished results.
29. **DeJuan, C., and R. Lagunas.** 1986. Inactivation of the galactose transport system in *Saccharomyces cerevisiae*. FEBS Lett. **207:**258–261.
30. **De Koning, W., and K. van Dam.** 1992. A method for the determination of changes of glycolytic metabolites in yeast on a subsecond time scale using extraction at neutral pH. Anal. Biochem. **204:**118–123.
31. **DeLaFuente, G., and A. Sols.** 1962. Transport of sugars in yeasts. II. Mechanism of utilization of disaccharides and related glycosides. Biochim. Biophys. Acta **56:**49–62.
32. **Dickson, R. C., and K. Barr.** 1983. Characterization of lactose transport in *Kluyveromyces lactis*. **154:**1245–1251.
33. **Egli, T., U. Lendenmann, and M. Snozzi.** 1993. Kinetics of microbial growth with mixtures of carbon sources. Antonie van Leeuwenhoek Int. J. Gen. Mol. Microbiol. **63:**289–298.
34. **Entian, K.-D.** 1980. A defect in carbon catabolite repression associated with uncontrollable and excessive maltose uptake. Mol. Gen. Genet. **179:**169–175.
35. **Entian, K.-D., and M. C. Loureiro-Dias.** 1990. Misregulation of maltose uptake in glucose-repression defective mutants of *Saccharomyces cerevisiae* leads to glucose poisoning. J. Gen. Microbiol. **136:**855–860.
36. **Fiechter, A., and W. Seghezzi.** 1992. Regulation of glucose metabolism in growing yeast cells. J. Biotechnol. **27:**27–45.
37. **Franzusoff, A., and V. P. Cirillo.** 1982. Uptake and phosphorylation of 2-deoxy-D-glucose by wild-type and single-kinase strains of *Saccharomyces cerevisiae*. Biochim. Biophys. Acta **688:**295–304.
38. **Franzusoff, A. J., and V. P. Cirillo.** 1983. Glucose transport activity in isolated plasma membrane vesicles from *Saccharomyces cerevisiae*. J. Biol. Chem. **258:**3608–3614.
39. **Fuhrmann, G. F., and B. Völker.** 1993. Misuse of graphical analysis in nonlinear transport kinetics by Eadie-Hofstee plots. Biochim. Biophys. Acta **1145:**180–182.
40. **Gancedo, C., and R. Serrano.** 1989. Energy yielding metabolism in yeast, p. 205–259. *In* J. S. Harrison and A. H. Rose (ed.), The yeasts, vol. 3, 2nd ed. Academic Press, Inc., New York.
41. **Gasnier, B.** 1987. Characterization of low- and high-affinity glucose transporters in the yeast *Kluyveromyces marxianus*. Biochim. Biophys. Acta **903:**425–433.
42. **Geurts, T. G. E., H. E. de Kok, and J. A. Roels.** A quantitative description of the growth of Saccharomyces cerevisiae CBS426 on a mixed substrate of glucose and ethanol. Biotechnol. Bioeng. **22:**2031–2043.
43. **Gonçalves, T., and M. C. Loureiro-Dias.** 1994. Aspects of glucose uptake in *Saccharomyces cerevisiae*. J. Bacteriol. **176:**1511–1513.
44. **Görts, C. P. M.** 1969. Effect of glucose on the activity and the kinetics of the maltose uptake system and α-glucosidase in *Saccharomyces cerevisiae*. Biochim. Biophys. Acta **184:**299–305.
45. **Harder, W., and L. Dijkhuizen.** 1976. Mixed-substrate utilization. p. 297–314. *In* A. C. R. Dean, D. C. Ellwood, C. G. T. Evans, and J. Melling (ed.), Continuous culture 6: applications and new fields. Ellis Horwood, Chichester, England.
46. **Hauer, R., and M. Höfer.** 1978. Evidence for interactions between energy-dependent transport of sugars and the membrane potential in the yeast *Rhodotorula gracilis* (*Rhodosporidium toruloides*). J. Membr. Biol. **43:**335–349.
47. **Herbert, D., R. Elsworth, and R. C. Telling.** 1956. The continuous culture of bacteria: a theoretical and experimental study. J. Gen. Microbiol. **14:**601–622.
48. **Höfer, M., and P. C. Misra.** 1978. Evidence for a proton/sugar symport in the yeast *Rhodotorula gracilis* (*glutinis*). Biochem. J. **172:**15–22.
49. **Kalina, V.** 1993. Dynamics of microbial growth and metabolic activity and their control by aeration. Antonie van Leeuwenhoek Int. J. Gen. Mol. Microbiol. **63:**353–373.
50. **Käppeli, O.** 1986. Regulation of carbon metabolism in *Saccharomyces cerevisiae* and related yeasts. Adv. Microbiol. Physiol. **28:**181–209.
51. **Ko, C. H., H. Liang, and R. F. Gaber.** 1993. Roles of multiple glucose transporters in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **13:**638–648.
52. **Kotyk, A., D. Michaljanicová, K. Veres, and V. Soukupová.** 1975. Transport of 4-deoxy- and 6-deoxy-D-glucose in baker's yeast. Folia Microbiol. **20:**496–503.
53. **Kreutzfeldt, C., and F. G. Fuhrmann.** 1984. Sugar transport in *Saccharomyces cerevisiae* H 1022. Swiss Biotechnol. **2:**24–27.
54. **Kruckeberg, A. L., and L. F. Bisson.** 1990. The *HXT2* gene of *Saccharomyces cerevisiae* is required for the high-affinity glucose transport. Mol. Cell. Biol. **10:**5903–5913.
55. **Lagunas, R.** 1993. Sugar transport in *Saccharomyces cerevisiae*. FEMS Microbiol. Rev. **104:**229–242.
56. **Lagunas, R., C. Dominguez, A. Busturia, and M. J. Sáez.** 1982. Mechanisms of appearance of the Pasteur effect in *Saccharomyces cerevisiae*: inactivation of sugar transport systems. J. Bacteriol. **170:**2654–2658.
57. **Lengeler, J. W.** 1993. Carbohydrate transport in bacteria under environmental conditions, a black box? Antonie van Leeuwenhoek Int. J. Gen. Mol. Microbiol. **63:**275–288.
58. **Lewis, D. A., and L. F. Bisson.** 1991. The *HXT1* gene product of *Saccharomyces cerevisiae* is a new member of the family of hexose transporters. Mol. Cell. Biol. **11:**3804–3813.
59. **Loureiro-Dias, M. C.** 1988. Movement of protons coupled to glucose transport in yeasts. A comparative study among 248 yeast species. Antonie van Leeuwenhoek Int. J. Gen. Mol. Microbiol. **54:**331–343.
60. **Lucero, P., M. Herweijer, and R. Lagunas.** 1993. Catabolite

inactivation of the yeast maltose transporter is due to proteolysis. FEBS Lett. **333**:165–168.
61. **Malpartida, F., and R. Serrano.** 1981. Proton-translocation catalyzed by the purified yeast plasma membrane ATPase reconstituted in liposomes. FEBS Lett. **131**:351–354.
62. **Matern, H., and H. Holzer.** 1977. Catabolite inactivation of the galactose uptake system in yeast. J. Biol. Chem. **252**:6399–6402.
63. **Meredith, S. A., and A. H. Romano.** 1977. Uptake and phosphorylation of 2-deoxy-D-glucose by wild type and respiration-deficient bakers' yeast. Biochim. Biophys. Acta **497**:745–759.
64. **Monod, J.** 1949. The growth of bacterial cultures. Annu. Rev. Microbiol. **3**:371–394.
65. **Mwesigye, P. K., and J. P. Barford.** 1993. Sucrose transport mechanism in *Saccharomyces cerevisiae*, p. 236. *In* W. A. Scheffers and J. P. van Dijken (ed.), Proceedings of the 16th International Specialized Symposium on Yeasts. Arnhem, The Netherlands. Pasmans, The Hague, The Netherlands.
66. **Needleman, R. B., D. B. Kaback, R. A. Dubin, E. L. Perkins, N. G. Rosenberg, K. A. Sutherland, D. B. Forrest, and C. A. Michels.** 1984. *MAL6* of *Saccharomyces cerevisiae*. A complex genetic locus containing three genes required for maltose fermentation. Proc. Natl. Acad. Sci. USA **81**:2811–2815.
67. **Neigeborn, L., and M. Carlson.** 1984. Genes affecting the regulation of *SUC2* gene expression by glucose repression in *Saccharomyces cerevisiae*. Genetics **108**:845–858.
68. **Nelson, N., and L. Taiz.** 1989. The evolution of H^+-ATPases. Trends Biochem. Sci. **14**:113–116.
69. **Nevado, J., M. A. Navarro, and C. F. Heredia.** 1994. Transport of hexoses in yeast. Re-examination of the sugar phosphorylation hypothesis with a new experimental approach. Yeast **10**:59–65.
70. **Noorman, H. J., J. Baksteen, J. J. Heijnen, and K. C. A. M. Luyben.** 1991. The bioreactor overflow device: an undesired selective separator in continuous cultures? J. Gen. Microbiol. **137**:2171–2177.
71. **Ongjoco, R., K. Szkutnicka, and V. P. Cirillo.** 1987. Glucose transport in vesicles reconstituted from *Saccharomyces cerevisiae*. J. Bacteriol. **169**:2926–2931.
72. **Osinga, K. A., R. F. Beudeker, J. B. van der Plaat, and J. A. de Hollander.** 1988. European patent 0 306 107.
73. **Osinga, K. A., A. C. H. M. Renniers, J. W. Welbergen, R. B. Roobol, and W. van der Wilden.** 1989. Maltose fermentation in *Saccharomyces cerevisiae*. Yeast **5**(Suppl.):207–212.
74. **Paardekooper, M., A. W. De Bruijne, J. Van Steveninck, and P. J. A. Van den Broek.** 1993. Inhibition of transport systems in yeast by photodynamic treatment with toluidine blue. Biochim. Biophys. Acta **1151**:143–148.
75. **Parulekar, S. J., G. B. Semones, M. J. Rolf, J. C. Lievense, and H. C. Lim.** 1986. Induction and elimination of oscillations in continuous cultures of *Saccharomyces cerevisiae*. Biotechnol. Bioeng. **28**:700–710.
76. **Peinado, J. M., A. Barbero, and N. van Uden.** 1987. Repression and inactivation by glucose of the maltose transport system of *Candida utilis*. Appl. Microbiol. Biotechnol. **26**:154–157.
77. **Peinado, J. M., and M. C. Loureiro-Dias.** 1986. Reversible loss of affinity induced by glucose in the maltose-H^+ symport of *Saccharomyces cerevisiae*. Biochim. Biophys. Acta **856**:189–192.
78. **Perlin, D. S., M. J. D. San Francisco, C. W. Slayman, and B. P. Rosen.** 1986. H^+/ATP stoichiometry of proton pumps from *Neurospora crassa* and *Escherichia coli*. Arch. Biochem. Biophys. **248**:53–61.
79. **Petrik, M., O. Käppeli, and A. Fiechter.** 1983. An expanded concept for the glucose effect in the yeast *Saccharomyces uvarum*: involvement of short- and long-term regulation. J. Gen. Microbiol. **129**:43–49.
80. **Pirt, S. J.** 1975. Principles of microbe and cell cultivation. John Wiley & Sons, Inc., New York.
81. **Postgate, J. R., and J. R. Hunter.** 1964. Accelerated death of *Aerobacter aerogenes* starved in the presence of growth-limiting substrates. J. Gen. Microbiol. **34**:459–473.
82. **Postma, E., A. Kuiper, W. F. Tomasouw, W. A. Scheffers, and J. P. van Dijken.** 1989. The competition for glucose between the yeasts *Saccharomyces cerevisiae* and *Candida utilis*. Appl. Environ. Microbiol. **55**:3214–3220.
83. **Postma, E., W. A. Scheffers, and J. P. van Dijken.** 1988. Adaptation of the kinetics of glucose transport to environmental conditions in the yeast *Candida utilis* CBS 621: a continuous-culture study. J. Gen. Microbiol. **134**:1109–1116.
84. **Postma, E., W. A. Scheffers, and J. P. van Dijken.** 1989. Kinetics of growth and glucose transport in glucose-limited chemostat cultures of *Saccharomyces cerevisiae*. Yeast **5**:159–165.
85. **Postma, E., and P. J. A. van den Broek.** 1990. Continuous-culture study of the regulation of glucose and fructose transport in *Kluyveromyces marxianus* CBS 6556. J. Bacteriol. **172**:2871–2876.
86. **Postma, E., C. Verduyn, A. Kuiper, W. A. Scheffers, and J. P. van Dijken.** 1990. Substrate-accelerated death of *Saccharomyces cerevisiae* under maltose stress. Yeast **6**:149–158.
87. **Postma, E., C. Verduyn, W. A. Scheffers, and J. P. van Dijken.** 1989. Enzymatic analysis of the Crabtree effect in glucose-limited chemostat cultures of *Saccharomyces cerevisiae*. Appl. Environ. Microbiol. **55**:468–477.
88. **Prior, C., H. Fukuhara, J. Blaisonneau, and M. Weselowski-Louvel.** 1993. Low-affinity glucose carrier gene *LGT1* of *Saccharomyces cerevisiae*, a homologue of the *Kluyveromyces lactis RAG1* gene. Yeast **9**:1373–1377.
89. **Ramos, J., K. Szkutnicka, and V. P. Cirillo.** 1989. Characteristics of galactose transport in *Saccharomyces cerevisiae* cells and reconstituted lipid vesicles. J. Bacteriol. **171**:3539–3544.
90. **Romano, A. H.** 1982. Facilitated diffusion of 6-deoxy-D-glucose in bakers' yeast: evidence against phosphorylation-associated transport of glucose. J. Bacteriol. **152**:1295–1297.
91. **Rouwenhorst, R. J., A. A. van der Baan, W. A. Scheffers, and J. P. van Dijken.** 1991. Production and localization of β-fructosidase in asynchronous and synchronous chemostat cultures of yeasts. Appl. Environ. Microbiol. **57**:557–562.
92. **Rouwenhorst, R. J., L. E. Visser, A. A. van der Baan, W. A. Scheffers, and J. P. van Dijken.** 1988. Production, distribution and kinetic properties of inulinase in continuous cultures of *Kluyveromyces marxianus*. Appl. Environ. Microbiol. **54**:1131–1137.
93. **Santos, E., L. Rodriguez, M. V. Elorza, and R. Sentandreu.** 1982. Uptake of sucrose by *Saccharomyces cerevisiae*. Arch. Biochem. Biophys. **216**:652–660.
94. **Schulz, B., and M. Höfer.** 1986. Utilization of lactose in non-respiring cells of the yeast *Debaryomyces polymorphus*. Arch. Microbiol. **145**:367–371.
95. **Seaston, A., C. Inkson, and A. A. Eddy.** 1973. The absorption of protons with specific amino acids and carbohydrates by yeast. Biochem. J. **134**:1031–1043.
96. **Serrano, R.** 1977. Energy requirements for maltose transport in yeast. Eur. J. Biochem. **80**:97–102.
97. **Spencer-Martins, I., and N. van Uden.** 1985. Catabolite interconversion of glucose transport systems in the yeast *Candida wickerhamii*. Biochim. Biophys. Acta **812**:168–172.
98. **Sreekrishna, K., and R. C. Dickson.** 1985. Construction of strains of *Saccharomyces cerevisiae* that grow on lactose. Proc. Natl. Acad. Sci. USA **82**:7909–7913.
99. **Szkutnicka, K., J. F. Tschopp, L. Andrews, and V. P. Cirillo.** 1989. Sequence and structure of the yeast galactose transporter. J. Bacteriol. **171**:4486–4493.
100. **Tkacz, J. S., and J. O. Lampen.** 1973. Surface distribution of invertase on growing *Saccharomyces* cells. J. Bacteriol. **113**:1073–1075.
101. **Van den Broek, P. J. A., J. Schuddemat, C. C. M. Van Leeuwen, and J. Van Steveninck.** 1986. Characterization of 2-deoxyglucose and 6-deoxyglucose transport in *Kluyveromyces marxianus*: evidence for two different transport mechanisms. Biochim. Biophys. Acta **860**:626–631.
102. **Van den Broek, P. J. A., C. C. M. Van Leeuwen, R. A. Weusthuis, E. Postma, J. P. Van Dijken, R. H. Karssies, and R. Amons.** 1994. Identification of the maltose transport protein of *Saccharomyces cerevisiae*. Biochem. Biophys. Res. Commun. **200**:45–51.
103. **Van den Broek, P. J. A., and J. Van Steveninck.** 1980. Kinetic analysis of simultaneously occurring proton-sorbose symport and passive sorbose transport in *Saccharomyces fragilis*. Biochim. Biophys. Acta **602**:419–432.
104. **Van den Broek, P. J. A., and J. Van Steveninck.** 1982. Kinetic

analysis of H^+/methyl β,D-thiogalactoside symport in *Saccharomyces fragilis*. Biochim. Biophys. Acta **693**:213–220.

105. **Van der Rest, M., Y. de Vries, B. Poolman, and W. N. Konings.** 1993. Maltose transport in membrane vesicles of *Saccharomyces cerevisiae*. p. 310. *In* W. A. Scheffers and J. P. van Dijken (ed.), Proceedings of the 16th International Specialized Symposium on Yeasts. Arnhem, The Netherlands. Pasmans, The Hague, The Netherlands.
106. **Van Dijken, J. P., and W. A. Scheffers.** 1986. Redox balances in the metabolism of sugars by yeasts. FEMS Microbiol. Rev. **32**:199–224.
107. **Van Leeuwen, C. C. M., E. Postma, P. J. A. van den Broek, and J. van Steveninck.** 1991. Proton-motive force-driven D-galactose transport in plasma membrane vesicles from the yeast *Kluyveromyces marxianus*. J. Biol. Chem. **266**:12146–12151.
108. **Van Leeuwen, C. C. M., R. A. Weusthuis, E. Postma, P. J. A. van den Broek, and J. P. van Dijken.** 1992. Maltose/proton cotransport in *Saccharomyces cerevisiae*. Comparative study with cells and plasma membrane vesicles. Biochem. J. **284**:441–445.
109. **Van Steveninck, J.** 1968. Transport and transport-associated phosphorylation of 2-deoxy-D-glucose in yeast. Biochim. Biophys. Acta **163**:386–394.
110. **Van Uden, N.** 1967. Transport-limited growth in the chemostat and its competitive inhibition: a theoretical treatment. Arch. Mikrobiol. **58**:145–154.
111. **Van Urk, H., E. Postma, W. A. Scheffers, and J. P. van Dijken.** 1989. Glucose transport in Crabtree-positive and Crabtree-negative yeasts. J. Gen. Microbiol. **135**:2399–2406.
112. **Verduyn, C., E. Postma, W. A. Scheffers, and J. P. van Dijken.** 1990. Energetics of *Saccharomyces cerevisiae* in anaerobic glucose-limited chemostat cultures. J. Gen. Microbiol. **136**:405–412.
113. **Verduyn, C., E. Postma, W. A. Scheffers, and J. P. van Dijken.** 1992. Effect of benzoic acid on metabolic fluxes in yeasts: a continuous culture study on the regulation of respiration and alcoholic fermentation. Yeast **8**:501–517.
114. **Verduyn, C., A. H. Stouthamer, W. A. Scheffers, and J. P. van Dijken.** 1991. A theoretical evaluation of growth yields of yeasts. Antonie van Leeuwenhoek Int. J. Gen. Mol. Microbiol. **59**:49–63.
115. **Verduyn, C., T. P. L. Zomerdijk, J. P. van Dijken, and W. A. Scheffers.** 1984. Continuous measurement of ethanol production by aerobic yeast suspensions with an enzyme electrode. Appl. Microbiol. Biotechnol. **19**:181–185.
116. **Von Meyenburg, H. K.** 1969. Energetics of the budding cycle of *Saccharomyces cerevisiae* during glucose limited aerobic growth. Arch. Mikrobiol. **66**:289–303.
117. **Walsh, M. C., H. P. Smits, M. Scholte, and K. Van Dam.** 1994. Affinity of glucose transport in *Saccharomyces cerevisiae* is modulated during growth on glucose. J. Bacteriol. **176**:953–958.
118. **Walsh, M. C., H. P. Smits, and K. van Dam.** Respiratory inhibitors affect incorporation of glucose into *Saccharomyces cerevisiae* cells, but not the activity of glucose transport. Submitted for publication.
119. **Wendell, D. L., and L. F. Bisson.** 1993. Physiological characterization of putative high-affinity glucose transport protein *HXT2* of *Saccharomyces cerevisiae* by use of anti-synthetic peptide antibodies. J. Bacteriol. **175**:7689–7696.
120. **Wéselowski-Louvel, M., P. Goffrini, I. Ferrero, and H. Fukuhara.** 1992. Glucose transport in the yeast *Kluyveromyces lactis*. I. Properties of an inducible low-affinity glucose transporter gene. Mol. Gen. Genet. **233**:89–96.
121. **Weusthuis, R. A., H. Adams, W. A. Scheffers, and J. P. van Dijken.** 1993. Energetics and kinetics of maltose transport in *Saccharomyces cerevisiae*: a continuous-culture study. Appl. Environ. Microbiol. **59**:3102–3109.
122. **Weusthuis, R. A., W. Visser, J. T. Pronk, W. A. Scheffers, and J. P. van Dijken.** 1994. Effects of oxygen limitation on sugar metabolism in yeasts: a continuous-culture study of the Kluyver effect. Microbiology **140**:703–715.
123. **Yao, B., P. Solliti, and J. Marmur.** 1989. Primary structure of the maltose-permease-encoding gene of *Saccharomyces carlsbergensis*. Gene **79**:189–197.